Nephronin: A series of compounds for treatment of infectious diseases and cancer.

Field of the Invention

The present invention relates novel compounds and their use in treatment and/or prevention of disease states.

Background of the Invention

In an era of increasing bacterial resistance to antibiotics and despite the immense efforts over the last decade to reduce the mortality from sepsis, and those associated with shock, the mortality rate remains as high as 50% (1) (2). Whilst endotoxin is the major mediator in the pathogenesis of septic shock, it should be noted that other bacterial products such as non-endotoxin bacterial cell wall peptidoglycan (PNG)(3), exotoxin of Gram positive and Gram negative bacteria alone or in conjunction with endotoxin can cause septic shock (4).

Lipopolysaccharide (LPS) or endotoxin is the major constituent of the outer membrane of Gram-negative bacteria (5). They cause a variety of pathophysiological effects in human and experimental animals (6). Bacteria release endotoxin when in circulation (7), causing the release of a number of pro-inflammatory cytokines that may lead to septic shock. It is noteworthy that the use of antibiotics results in bacterial death, and release of endotoxin (8; 9). This in turn may exacerbate the problem by overwhelming the immune system, leading to shock (10).

Lipid A is the most conserved region of LPS and is responsible for virtually all of the toxic activities of LPS. LPS stimulates a range of cells, most notably macrophages (MØ), to synthesise cytokines including interleukin-1α (IL-1α), interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α) (11-13). Pico-molar concentrations of LPS have been shown to induce TNF-α production 30-60 minutes after intravenous injection of LPS (14). TNF-α and/or IL-1 play an important role in the development of septic shock (10; 15). Further, these cytokines were shown to mimic the whole spectrum of LPS toxicity in animal models (16-18), and that they may have synergistic lethal effect in septic shock (19).

It has been shown that certain serum proteins enhance the cytokine stimulation ability of LPS (20) (21). Tobias has reported the discovery of a 60KDa acute phase LPS binding serum glycoprotein synthesised by hepatocytes (22). This LPS-binding protein (LBP) binds tightly to the lipid A region of LPS (23). LBP has been shown to have a high amino acid similarity with

bactericidal/permeability-increasing protein (BPI) (24). However, despite their similarities in amino acid sequence and their ability to bind to LPS, LBP and BPI have significantly different biological consequences (25). LBP greatly enhances the biological effects of LPS, by reducing the threshold for LPS's effect by 1000 fold, and the rate of cytokine production is also markedly accelerated (26). In contrast, BPI inhibits the ability of LPS to stimulate cells. Furthermore, when in contact with intact bacteria, BPI has a bactericidal activity, whereas LBP acts as an opsonin. Although the levels of LBP increase from 3-10μg/ml to as much as 200μg/ml during Gram negative sepsis, the levels of BPI rarely increase above normal.

LBP seem to function as a lipid transfer protein for the delivery of endotoxin as LPS-LBP complex to CD14 (23). CD14 is a 50-55KDa glycosylphosphatidyl inositol (GPI)-anchored membrane glycoprotein expressed on myeloid cells (membrane CD14; mCD14) (27) or a soluble form lacking the GPI anchor (soluble CD14; sCD14) (28).

Lee *et al* have clearly shown the importance of CD14 in controlling LPS responses and have provided evidence that LPS exerts its effects via CD14-dependent pathways (29). The same group has also provided evidence suggesting that binding of endotoxin to cell surface CD14 is followed by subsequent interactions with additional membrane protein(s) that initiate trans-membrane signalling (30). It is of interest that mCD14, a GPI-anchored protein that does not directly communicate with the cell interior, can mediate trans-membrane signalling.

Despite the huge amounts of money spent in developing new therapeutic agents, including antibodies against endotoxin (31), or against TNF-α (32), and synthetically produced antagonists of endotoxin (33; 34), currently there is no effective remedy. Polymyxin B, a cationic cyclic peptide antibiotic, which inhibits the biological effects of endotoxin through its high affinity binding to lipid A, has also been tested as a potential therapeutic candidate (35). However its use is limited by its high toxicity. Unfortunately none of these agents have proved particularly beneficial.

The young and geriatrics are more susceptible to bacterial infection with high mortality. These failed trials demonstrate the pressing need to develop effective, bioavailable therapeutics. In recent years however, the discovery of biologically active compounds from the urine of women in early pregnancy, in particular that of an anti-HIV factor from the urine of such individuals (36) signifies the importance of this work for new drug development. The present inventor's work on steroid responsive nephrotic syndrome (SRNS) has led to the discovery of a low molecular weight,

non-proteinaceous compound that specifically binds to heparin and heparan sulphate and neutralises endotoxin. This compound has been named "Nephronin".

Infection and Heparan sulphate

Heparan sulphate serves as a receptor for adherence of herpes simplex viruses (HSV), chlamydia trachomatis, Neisseria gonorrhoeae, and indirectly human immunodeficiency virus (37). HSV causes many disease states, including mucosal lesions, encephalitis or disseminated infection in immunocompromised host. These diverse clinical manifestations reflect the capacity of the virus to infect both epithelial and neuronal cell types (38). Previous studies have shown that the first step in herpes virus infection is attachment to heparan sulphate molecule on the surface of cells (39-41). Given the fact that Nephronin specifically binds to heparin and heparan sulphate and its ability to neutralise Gram negative endotoxin suggests that Nephronin may be a prime candidate for inhibiting HSV infection and other pathogens that employ similar mechanism for infection. Furthermore, recent studies have placed and important role for heparan sulphate in cell-cell interaction, metastasis and tumour growth. Therefore the potential for Nephronin as an anti metastasis, anti-cancer drug is apparent.

Summary of the Invention

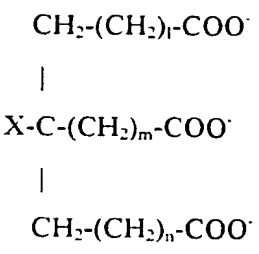
In a first aspect the present invention consists in a non-proteinaceous compound isolatable from the urine of patients suffering from steroid responsive nephrotic syndrome, the compound having the following characterising features:

(i) a molecular weight less than 1 KDa;

(ii) binds specifically to heparin and heparan sulphate but not other glycosaminoglycans; and

(iii) inhibits LPS induced production of TNF-α and/or IL-1α.

In a second aspect the present invention consists in a compound of the general formula:-



in which l,m and n may be the same or different and integers of 0 to 10; and

X is R-CO-O-, R-O-PO_2-O-, R-O-SO_2-O-, R-CO-NH-, R-CO-, R-O- or monosaccharide or oligosaccharide including amide substituted saccharides; in which R is a saturated, unsaturated, branched or cyclic carbon chain of up to 32 carbon atoms which optionally contains one or more hydroxyl groups, carbonyl groups, carboxylic acids groups, amino groups, phosphate groups or sulphate groups or combination thereof;

and pharmaceutically acceptable salts and derivatives therof.

In a preferred embodiment of this aspect of the present invention the X is R-CO-O- in which R is CH_3 or $CH_3(CH_2)_f$ where f is an integer from 1 to 18, preferably 1 to 8. It is further preferred that R is selected from the group consisting of CH_3, CH_3CH_2 $CH_3(CH_2)_2$ $CH_3(CH_2)_4$ and $CH_3(CH_2)_6$.

In a third aspect the present invention consists in a composition comprising the compound of the first or second aspects of the present invention and a pharmaceutically acceptable carrier.

In a fourth aspect the present invention consists in a method of treating, preventing, or reducing the risk of Gram negative septic shock or another disease state involving elevated cytokine levels in a subject, the method comprising administering to the subject an effective amount of the composition of the third aspect of the present invention.

In a fifth aspect the present invention consists in a method of treating, preventing, or reducing the risk of viral infection in a subject, the method comprising administering to the subject an effective amount of the composition of the third aspect of the present invention.

It is preferred that the virus is Herpes virus or HIV.

In a sixth aspect the present invention consists in the use of the compound of the present invention in medicine.

In a seventh and eighth aspect the present invention consists in the use of the compound of the present invention in the preparation of a medicament for use in the treatment, prevention or reducing the risk of Gram negative septic shock or another disease state involving elevated cytokine levels and the preparation of a medicament for use in the treatment, prevention or reducing the risk of viral infection.

In a ninth aspect the present invention consists in a method of treating, preventing, or reducing the risk of metastases or angiogenesis in a subject, the method comprising administering to the subject an effective amount of the composition of the third aspect of the present invention.

In a ninth aspect the present invention consists in the use of the compound of the present invention in the preparation of a medicament for use in the treatment, prevention or reducing the risk of of metastases or angiogenesis.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following Examples.

Figure Legends

Fig. 1 Dose-dependent inhibition of LPS-induced TNF-α and IL-α by four different preparations of compounds of the present invention. The indicated amount of each preparation was added to chambers of 24 well culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. LPS was added to the cell suspension a few minutes prior to dispensing the cells into the wells of the tissue culture plates. The cells were harvested at 24 and 48 post-stimulation and the concentration of the cytokines measured by sandwich ELISA.

A) Dose-dependent inhibition of LPS induced TNF- α, 24h post-stimulation

B) Dose-dependent inhibition of LPS induced IL- 1α, 24h post-stimulation

C) Dose-dependent inhibition of LPS induced TNF- α 48h post-stimulation

d) Dose-dependent inhibition of LPS induced IL- 1α, 48h post-stimulation

Similar results were also obtained at 72h post-stimulation

Fig. 2 Dose dependent inhibition of human platelet heparanase activity by the preparations of Nephronin. Preparations 1-5 refer to preparations of compounds of the present invention with

increasing carbon chain length. Preparation 6 is a control preparation. Blank (Blk) refers to a control for the enzyme reaction.

Fig. 3 Two preparations of compounds of the present invention were tested for their ability to inhibit HSV infection of human keratinocytes. The HSV inoculum used contained 15 plaque forming units (PFU) of HSV per cell. 5 µl of each preparation of compound was added to chambers of 12 well tissue culture plate containing the keratinocytes in a final volume of 2ml tissue culture media.

Fig. 4 Inhibition of axonal spread of HSV1 to epidermal cells. An *in vitro* model consisting of human dorsal root ganglia (DRG) neurones and autologous epidermal cells in two separate chambers to study anterograde axonal transport of HSV1 was used. 5 µl of compounds of the present invention was added at the indicated times to the epidermal cell (ECs) side of the chambers in a final volume of 2ml. HSV1 inoculum was added to the DRG side of chambers.

Materials and Methods.

Chemicals

All chemicals were purchased from Sigma Aldrich, unless otherwise stated.

Purification of Nephronin

Urine, from SRNS patients in relapse, was collected in untreated clean 24-hour collection bottles. The urine bottles were then stored at -20°C until used. The protein content of urine samples was determined using Multistix SG (Bayer Diagnostic UK LTD. Basingstoke. Hampshire. UK). Urine samples with protein content of 3+ were used for the purification of the factor.

Up to 2 litres of urine was thawed, concentrated and dialysed extensively against milli-Q water using an Amicon spiral cartridge concentrator/filtration system with a cut-off of 10,000 dalton. The concentrated urine was then freeze-dried and the lyophilised powder resuspended in 0.1M ammonium acetate buffer pH 9.0. The pH of the sample was then adjusted to pH 9.0 with a solution of 10% ammonium hydroxide. The resuspended sample was centrifuged at 30,000g for 20min. at 4°C, prior to column chromatography.

The clear supernatant was loaded onto a 300 ml Fast-Q Sepharose column (Pharmacia. St Albans, Herts, UK) previously pre-equilibrated with 0.1M ammonium acetate buffer pH 9.0 at 4°C.

The column was run isocratically at 4°C. Material eluted unbound to the column under these conditions were pooled and freeze-dried. The bound material was eluted from the column with 2M NaCl in the same buffer and discarded.

The freeze-dried material was then resuspended in 20-50 ml of 0.1M sodium citrate pH 3.5. The pH of the sample was adjusted to pH 3.5 with 1M citric acid. The resultant cloudy solution was centrifuged at 30,000g for 20min at 4°C and the clear supernatant was then loaded onto a Hiload 26/10 S-Sepharose high performance column, previously pre-equilibrated with 0.1M sodium citrate pH 3.5. The sample was loaded onto the column at 2ml/min, followed by isocratic elution of unbound material by 0.1M sodium citrate until the base line was achieved. The bound material was eluted with step elution of 10, 25 and 100% of the same buffer containing 2M NaCl and 5 ml fractions were collected. Fractions eluted with the 25% salt step (0.5 M NaCl in 0.1 sodium citrate) were pooled and dialysed extensively against milli-Q water.

The pooled material was then freeze-dried and the lyophilised material was resuspended in 0.1M-ammonium acetate pH 9.0. The pH was adjusted to pH 9.0 with a solution of ammonium hydroxide and was then loaded onto a Q-Sepharose column. Under these conditions, the majority of the material bound to the column. After loading the column with the sample, the column was washed with at least 4-column volume of water. Finally the bound material was eluted as a single broad peak with 3.0% formic acid. This material was freeze-dried several times to remove the formic acid buffer. The lyophilised material was resuspended in water and pH adjusted to neutral with a 10% solution of ammonia.

This material appeared to be non-proteinaceous, since standard protein estimation assays failed to show the presence of any protein. This was further confirmed by the fact that this material did not absorb at 280nm. Spectral analysis showed maximum peak at approx. 205-215nm.

Cellulose acetate electrophoresis

Flat-bed cellulose acetate electrophoresis was used for the detection of material that might bind to different glycosaminoglycans and inhibit their migration. Cellulose acetate (Sartorius Limited, Epsom, Surrey, UK.) sheets of 15cm by 7.5cm were pre-soaked in the running buffer, 0.1M Barium acetate pH 6.9. The excess buffer was removed from the acetate sheet by placing the sheet between two sheets of tissue paper. The sheet was then positioned in the electrophoresis tank and 0.1 units of

glycosaminoglycans, in a volume of up to 1 µl, was loaded onto numbered positions no more than 2 mm in diameter on the cellulose acetate sheet and dried under the heat of a lamp.

To test the binding ability of the samples (factor) to GAGs, in particular heparin, generally up to 2µl of each sample was loaded on to the GAG spot and dried under the heat of a lamp. However, in cases where the samples were particularly dilute, up to 10µl of each sample was loaded onto each spot in 2µl aliquot. The spots were dried under a lamp prior to further loadings.

Cellulose acetate electrophoresis was performed at 60 volts for 3 hours towards anode.

The GAGs on the cellulose acetate sheet were then visualised by staining with a solution of Alcian Blue 8GX (0.05% Alcian blue in 50mM sodium acetate pH 5.8 containing 50mM $MgCl_2$). The excess dye was washed from the cellulose acetate sheet with a solution of 5% acetic acid. The cross-reactivity of the GAGs with the samples was assessed by the inhibition in migration compared with the migration of untreated corresponding GAG.

Nephronin was shown to bind specifically to Heparin and heparan sulphate but not chondroitin and dermatan sulphate. This is demonstrated by the inhibition in migration of these glycosaminoglycans. The inhibition in migration of heparin and heparan sulphate is ascribed to specific interaction of Nephronin with these molecules, rather than on the basis of charge, since all these molecules are highly negatively charged.

Endothelial cells.

Endothelial cells were obtained from human umbilical veins by collagenase (0.1%) digestion as previously described (42) and modified by Klein et al (43).

Isolation of PBMC

Whole blood (10 ml) from SRNS patients and healthy volunteers was collected into 50 ml sterile Greiner tubes (Greiner Laboratory, Dursley, Glos. UK) containing 1ml sterile sodium citrate / citric acid (3 parts 0.1M sodium citrate, 2 parts 0.1M citric acid) anticoagulant and diluted 1:3 with sterile PBS. The 33.0 ml PBS/blood/anticoagulant was layered onto 16.5 ml Ficoll/hypaque, Pharmacia, St Albans, herts, UK) and centrifuged at 1800g for 30min at 4°C. The cell layer at the interface between ficoll and plasma was removed, diluted 1:5 v/v with ice cold PBS and centrifuged at 750g for 10min at 4°C. The pelleted cells were washed once more with cold PBS and the final cell

pellet was resuspended in 5 ml of RPMI 1640 culture medium (Gibco, Paisley, Scotland, UK) containing 10% heat inactivated foetal calf serum (FCS) (Sigma chemical company, Poole, Dorset, UK). The cells were then aliquoted in 8-well culture plates (Nunc, Paisley, Scotland, UK) for 45min at 37°C in incubators supplied with air, 5% CO2, to allow macrophages to adhere to the plastic. Following adherence, the cells suspension were removed and washed with pre-warmed RPMI 1640 at 37°C and maintained in the same culture medium supplemented with 50 units per ml of IL-2 (Zymed Laboratory INC., distributed by Cambridge Bioscience, Cambridge, UK.).

Histochemical studies

Endothelial cells were grown to confluence on gelatinised cover slips. Once confluence was achieved the cover slips were removed and the cells were washed with phosphate buffered saline (PBS) at 37°C. The cells were then fixed with 4% paraformaldehyde in PBS solution. For specific staining of heparan sulphate, the staining procedure was performed at pH 1.9. Under these conditions all proteinaceous material is positively charged, whilst heparan sulphate moieties remain negatively charged. 5nm gold-conjugated poly-L-lysine probe particles (Biocell Research Laboratories, Cardiff, UK) were used for specific staining of heparan sulphate, based on charge interaction, essentially as described by the manufacturers. The cells were counter stained in Meyer's Haematoxylin for 1min prior to mounting in Aquamount (BDH, UK).

Coagulation studies

Extrinsic, UPTT and intrinsic, KPTT were performed as directed by the suppliers (Diagnostic Reagents Limited, Thames, Oxon, UK). Commercially available Kits for the determination of heparin like molecules based on the antithrombin III activity for the inhibition of thrombin formation were used for the measurement of the factor, as directed by the suppliers (KABI Diagnostica. Supplied by Quadratech, Surrey, UK).

SDS-PAGE

Polypeptides were separated by SDS-PAGE [Laemmli, 1971 #72], electroblotted on to nitrocellulose sheets (44). The blotted protein bands were visualised on the nitrocellulose sheet with

amido black staining solution [0.2% (w/v) amido black, 25% (v/v) isopropanol, 10% acetic acid], and de-stained in the same solution without dye.

The protein bands were sent to Ludwig Institute for Cancer research, Riding house street, London, UK, for end terminal amino acid sequencing.

Thin layer chromatography and staining for peptides, lipids, carbohydrates and oligosaccharides were performed as previously described (45).

Protein estimation was carried out using the Bradford method (46).

Characterisation of Nephronin

Purification of the hypothesised circulatory highly cationic protein clearly showed that this protein was of immunoglobulin origin and that its existence was an artefact. However, there is a considerable amount of evidence to suggest that the proteinuria in nephrotic syndrome is due to a reduction in the negative charge of heparan sulphate on the glomerular capillary wall, brought about by a circulatory factor. During the search for the cationic protein, a number of assay methods for the detection of heparin binding molecules were developed.

A method for detecting Nephronin, was to use a one dimensional cellulose acetate electrophoresis, that is often used in the characterisation of GAGs (47). However, the data obtained using cellulose acetate electrophoresis must be treated cautiously, since false positive data can be obtained with high concentrations of salts, including sodium citrate.

Using the modified cellulose acetate electrophoresis for the detection of a heparin binding factor, a factor was isolated from the urine of children with SRNS, that binds to and inhibits the migration of glycosaminoglycan, heparin. Further studies for the specificity of this factor for binding other GAGs, clearly showed that this factor only inhibited the migration of heparin and heparan sulphate but not other glycosaminoglycans on cellulose acetate electrophoresis.

Nephronin was found to be highly negatively charged based on its tight binding to anion exchange columns (Q-column) at neutral pH. As well as the cellulose acetate electrophoresis for its detection, thin layer chromatography was performed for the identification of various components associated with Nephronin during the purification procedure. Staining of this factor for peptides, carbohydrates and lipids at various stages of purification clearly demonstrated that Nephronin was tightly bound to lipids. The lipid contamination proved to be a great obstacle in the purification of

Nephronin, and for a long period the heparin/heparan sulphate binding ability of Nephronin was ascribed to the lipid entity.

Standard chloroform/methanol extraction for the separation of the lipid entity, resulted in a huge reduction in the activity of the factor, with the active material in the aqueous layer free from the lipid entity.

Nephronin was then further purified by a number of other chromatographic techniques and the active fractions retained on the basis of their binding to heparin and inhibition of its migration on cellulose acetate electrophoresis.

During the purification of Nephronin, Amicon stirred cell ultrafiltration with membranes of 30, 10 and 1kDa cut off were used. These studies indicated that Nephronin had a relatively large molecular weight. However, under reducing conditions, Nephronin was found to pass through membranes with a cut of 1kDa. Gel filtration studies under reducing conditions also indicated a molecular size of less than 2KDa.

Thin layer chromatography and staining for carbohydrates, lipids and oligosaccharides, as well as phenol-sulphuric acid assay (48) for carbohydrate determination were negative. Pronase digestion of the factor also had no effect on its heparin binding, inhibition of its migration on cellulose acetate electrophoresis.

Cross reactivity of Nephronin with heparan sulphate on the endothelial cells

Although the specificity of Nephronin for heparin and heparan sulphate was shown using the cellulose acetate electrophoresis method, it was important to establish the effects of this material on endothelial cells, since the GAG coating the endothelial cells has been shown to be almost exclusively heparan sulphate (49).

The binding of Nephronin to heparan sulphate on endothelial cells was studied on paraformaldehyde pre-fixed cells and then viable cells.

1. Effect of the factor on fixed cells.

Exposure of paraformaldehyde fixed endothelial cells to isolated Nephronin, diluted in PBS, showed a considerable reduction in the number of anionic sites. This demonstrates that the

treatment of endothelial cells with Nephronin abolishes the characteristic staining for heparan sulphate on the untreated endothelial cells.

2. Effect of the factor on viable cells.

In order to test the effect of the factor on cell surface heparan sulphate of viable endothelial cells, the factor was added to the culture medium (up to 5% v/v). Initial experiments showed that exposure of the viable cells to the factor caused the dissociation of the cells from the substratum. To minimise the dissociation of the cells from the substratum, the incubation period was reduced from 1 hour to 10min. The cells were then stained for surface anionic sites as above.

3. Effect of the factor on the viability of the endothelial cells.

To assess the binding of Nephronin to heparan sulphate on viable endothelial cells, Nephronin was added to confluent endothelial cells. In the initial experiments the endothelial cells were exposed to Nephronin for one hour as described above. However, it was found that during this period almost all of the endothelial cells were detached from the culture plate. To investigate whether Nephronin had caused cell death, resulting in the separation of cells from the substratum, the culture medium containing the cells was removed and centrifuged at 1000rpm for 10min. The pelleted cells were resuspended in cold PBS and washed twice with cold PBS. Trypan blue staining of the cells showed that over 95% of the cells were viable.

To continue the investigation for the binding of Nephronin with the cell surface heparan sulphate, the incubation time was reduced to 15 minutes and the amount of Nephronin added was reduced to 2.5% v/v of the culture volume. A considerable reduction in the GAG staining of the cells treated with the factor was observed.

Cross-reactivity of Nephronin with heparin in coagulation studies.

Having established Nephronin's ability to bind to heparin and heparan sulphate, it was important to establish whether this material had any effect on blood coagulation.

Both extrinsic, UPTT and intrinsic, KPTT coagulation systems were used. Nephronin was found to prolong coagulation time in much the same way as heparin. Furthermore, the anti-coagulant effect of Nephronin was found to be synergistic with heparin.

To further investigate this relationship with heparin, a commercially available chromogenic kit for the measurement of heparin like molecules, based on the acceleration of antithrombin III activity for the inhibition of thrombin formation was used (50; 51). Once again, it was found that Nephronin behaved in much the same way as heparin and in synergy with heparin. These findings suggest that Nephronin may be able to self-polymerise, since activation of antithrombin III requires at least a pentasaccharide sequence of heparin (52).

Identification of the cells involved in the production (secretion) of Nephronin

Although the pathogenesis of SRNS is still unknown, it is well known that patients with SRNS go into relapse with upper respiratory and minor infections. As mentioned earlier the loss in immune regulation is thought to play an important part in the pathogenesis of the disease.

To test whether the immune cells from SRSN patients during relapse produce (secret) Nephronin without mitogen stimulation of the cells, and whether it is possible to induce immune cells from normal subjects to produce Nephronin, PBMC from SRNS patients and normal subjects were isolated.

Production of Nephronin by human lymphocytes.

PBMC isolated from SRNS patients were maintained in RPMI 1640 culture medium containing 10% v/v heat inactivated FCS and 50units/ml of IL-2. The culture medium was removed at 24, 48 or 72 hour time periods and stored frozen at -70°C until use.

The culture mediums were then processed analytically for the purification of Nephronin. To assess the effect of the Nephronin on unstimulated blood cells, PBMC were also isolated from healthy volunteers. The PBMC were maintained as above except that on these occasions the culture media were supplemented with 10% SRNS patient's plasma. For control studies PBMC isolated from healthy subjects were cultured as above and supplemented with 10% normal plasma. Plasma from SRNS and healthy subjects were prepared by mixing 10 ml of whole blood with 1ml of sterile sodium citrate / citric acid (3 parts 0.1M sodium citrate, 2 parts 0.1M citric acid) as anticoagulant. The cells were removed by centrifugation at 850g for 10min. The clear plasma was removed and stored in small aliquots at -70°C. The pelleted cells were usually used for other related experiments.

The data presented in Table 3 clearly demonstrates that, lymphocytes isolated from SRNS patients during relapse are capable of producing Nephronin. Furthermore, addition of patients plasma, but not plasma from normal subjects, to cultures of lymphocytes from normal subjects also induced these cells to produce Nephronin. These results suggests

1. Lymphocytes from SRNS patients during relapse produce Nephronin.
2. There is a circulatory factor in the plasma of these patients that can stimulate lymphocytes isolated from normal subjects, to produce Nephronin.

The fact that the immune cells from SRNS patient during relapse produce Nephronin, indicates the importance of this compound as part of the immune response to infection. However, the lack of immune regulation, as suggested by many workers in the field, may be responsible for the onset of nephrotic syndrome.

Isolation of Nephronin from urine of meningitis patients.

The studies above indicated that the immune cells in response to infection could produce Nephronin. To test this hypothesis, urine from three children with meningitis and two normal children were processed for analytical isolation of Nephronin. Nephronin was isolated from urine of two of the meningitis patient who had survived the disease. The urine of the patient that did not contain Nephronin did not survive the disease.

Nephronin was not isolated from the urine of normal children.

Endotoxin neutralising ability of Nephronin.

Because of the anionic nature of Nephronin and its ability to bind to lipids, it was postulated that this factor might behave as a cationic detergent. Furthermore, the fact that Nephronin was also isolated from urine of children with meningitis suggested that this factor is produced in response to infection. Consequently, Nephronin was thought to behave similarly to that of the cationic antibiotic, polymyxin.

Limulus Amoebocyte Lysate (LAL), utilises the E.coli endotoxin for the activation of a pro-enzyme which then hydrolyses a substrate to yield a chromogenic product. One such system was used to demonstrate the possible anti-endotoxin ability of Nephronin.

Table 1 show a representative set of data for the inhibition of endotoxin by Nephronin.

Agglutination of Gram-negative bacteria.

The fact that Nephronin inhibited LPS by the LAL assay, indicated that this compound might bind to LPS molecule and neutralise its effect by direct physical binding. To test whether, binding of Nephronin to LPS had an effect on bacterial agglutination, a number of Gram negative and Gram positive bacteria were used to test this hypothesis. Furthermore, since LPS molecule is not always exposed in different strains of Gram negative bacteria, the agglutination of Nephronin on heat and/or penicillin killed bacteria was also test. Table 2 shows the agglutination of Gram negative but not Gram positive bacteria by Nephronin. It is noteworthy that in the cases of heat and penicillin killed bacteria, agglutination occurred very quickly, whereas where viable bacteria were used, this was not always readily apparent.

Physicochemical Characterisation of Nephronin

Preliminary characterisation of Nephronin suggested the absence of peptide bonds involved in its heparin binding ability, since this activity is not affected by treatment with 6M HCl and heating at 105°C for 24h. Furthermore, thin layer chromatography and staining for carbohydrates, lipids and oligosaccharide, as well as phenol-sulphuric acid assay for carbohydrate determination were negative. Pronase digestion of Nephronin also seems to have no effect on its heparin binding and inhibition of its migration on cellulose acetate electrophoresis.

Elemental analysis tests for Carbon, Hydrogen, Phosphorous and sulphur of Nephronin, only showed the presence of 37.04% carbon and 3.74% hydrogen.

Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS) of the isolated Nephronin were inconclusive. Extensive NMR studies clearly showed the presence of citrate molecule. Although considerable washing steps were performed during the chromatographic steps, the presence of citrate was not surprising since it was used as one of the buffering systems. However, characteristic citrate profile (doublets) was often found to be shifted from 2.7 to approx. 3.7, suggesting a citrate product, most likely an ester on the 2-hydroxyl group of the citrate molecule. The data from FAB-MS indicated stable molecular weight of approximately 500 mass units for the factor with shedding of masses corresponding to one and two carbon fragments.

The factor was subjected to reverse phase column chromatography using a c18 column with a liner gradient of 0-100% acetonitrile. Essentially there were four pooled fractions. These fractions were subjected to mass spectroscopy. The data showed the presence of fatty acids of saturated C18 and C16 and an unsaturated C16 fatty acyl groups.

These data were inconclusive for determination of an exact molecular structure, nevertheless these studies indicated that the factor was an ester of citrate esterified with heterogeneous group of fatty acids on the 2-hydroxyl group of the citrate molecule.

However, based on the information available, a plausible structure for Nephronin was hypothesised. Methodology for synthesising Nephronin-like compounds was then proposed, and successful synthesis of of these compounds achieved. The synthesis procedure described for the new compounds of the present invention is not limiting and only serves to demonstrate the synthesis of five different, biologically active compounds.

Establishment of a biological system

To test the effect of the compounds of the present invention in a biological system, the products from different synthesis were tested on human lymphocytes, isolated from blood buffy coat (concentrated White Blood Cell preparation, supplied by the Blood Bank). Briefly, 10ml of buffy coat was mixed with 30ml of PBS. This mixture was then layered on 10ml of ficoll in a 50ml falcon tube, without mixing the two layers. The tubes were then centrifuged at 2700rpm (1800g) for 30min. The lymphocytes at the interface were gently removed by sterile plastic pipette into a clean tube. The cell suspension was diluted with PBS at least 4 times. The tube containing the cells were inverted and the centrifuged at 1700rpm for 15min. The cell pellets were resuspended in 10ml of PBS and centrifged as before. The rsulting cell pellet were washed once more with PBS. Finally the cells were ressupended in complete RPMI containing 10% Foetal Calf Serum. The lymphocytes were counted using a haemocytometer and the number of cells were adjusted to $1.5x10^6$ cells per ml. Lymphocytes were stimulated with 1ng/ml of endotoxin (*E.coli*, serotype 055:B5, Sigma-Aldrich chemical company) in the presence and absence of the synthesised compounds. For positive controls, a number of other biological stimulators were also used. These included, PHA, Concanavalin A, streptococcal bacterial membrane protein (M protein) and enterotoxins (superantigens) SpeA and SpeB.

Sandwich ELISA for the measurement of mitogen induced cytokines was established. Sandwich ELISA for TNF-α, and IL-1α was routinely used for the measurement of these cytokines in the culture supernatant of the activated cells post-stimulation. Cells were harvested at 24, 48 and 72 hours post-stimulation and the culture supernatant's were used in the determination of the cytokine levels.

Specific antibodies were purchased from a number of suppliers; mouse monoclonal and rabbit polyclonal antibodies against human TNF-α were purchased from Genzyme, USA, supplied by Lab Supply, Australia. Mouse monoclonal and rabbit polyclonal antibodies against human IL-1α, IL-4, IL-6 and interferon-γ (IFN-γ) were purchased from Endogen, USA, supplied by CSL Biosciences. Horseradish peroxidase conjugated anti-rabbit antibodies were purchased from Zymed, USA.

Cytokines used in the standard curves were purchase from Peprotech INC, supplied by Australian Laboratory Services Ltd.

Briefly, in the case of TNF-α, monoclonal antibodies were resuspended in freshly prepared 0.06M carbonate/bicarbonate coating buffer pH 9.6, at a concentration of 0.5mg/ml antibodies. 100µl of this mixture was added to each well of a 96 well ELISA plates. The plates were tightly wrapped in gladwrap to prevent evaporation and the plates were placed in a 37°C incubator for threes hours. After this time, the plates were washed with PBS buffer containing 0.05% Tween-20, using a wash bottle to fill each well with the buffer, followed by inverting the tube to empty the wells. After the washing procedure the excess buffer from the wells were removed by tapping the plates on a towel whilst inverted. To reduce non-specific binding, 100µl of PBS/Tween Buffer containing 0.1% casein was added to each well. The plates were then wrapped in gladwrap and placed in an incubator at 37°C for 60min. The plates were washed three times as before and the culture supernatants from the treatment of the lymphocytes with mitogens including endotoxin in the presence and absence of preparations of the compounds of the present invention were added to the test wells. Each sample was tested at three different dilutions of 4, 8 and 16 in PBS/Tween-20 buffer containing 0.01% casein. The plates were then incubated at 4°C over night. A standard curve was produced, using recombinant human TNF-α. 100µl of this was used from 500pg/ml to 2pg/ml. For negative control, just buffer was added to 4 wells. The plates were washed 6 times with PBS Tween-20. 100µl of 1µg/ml rabbit polyclonal antibodies to human TNF-α was then added to each

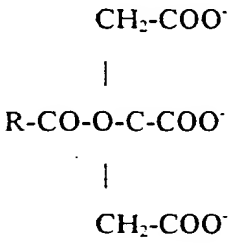
well and incubated at RT for two hours. The plates were washed 6 times with PBS/Tween-20. 100µl of horseradish peroxidase conjugated anti-rabbit antibodies, diluted 1500 in PBS/Tween-20 buffer, was added to each well. The plates were incubated for a further 2 hours at 4°C. Finally the plates were washed as before. The plates were dried by tapping on the towel and the developed with the addition of 100µl of substrate solution, containing 0.4mg orthophenyl diamine (purchased as tablets from Sigma) in citrate/phosphate buffer pH 5.5. The reaction was stopped with the addition of 100µl of 2M sulphuric acid. The plates were then read using a Biorad ELISA plate reader using a 495nm filter. The concentration of the TNF- α in each samples was estimated from the standard curve. Using this methodology, the assay had a sensitivity of up to 5pg/ml.

Similar methodology was used to establish an ELISA assay for IL-1α with similar sensitivity.

Synthesis of Nephronin-like compounds.

The hypothesised structure for Nephronin assumes that this compound is surfactant like in structure with specificity towards certain sugar moieties, in particular heparin/heparan sulphate, hence an anionic molecule that behaves like a cationic detergent. Taking this into consideration, one can then visualise Nephronin being able to inhibit LPS.

NMR studies on the isolated Nephronin had demonstrated the presence of citrate (citric acid). The hypothesised structure for Nephronin was;



in which R is an alkyl group.

Method of synthesis

5.7ml of glacial acetic acid was added to 3g of tri-sodium citrate (Na3Citrate). The mixture was quickly and vigorously mixed and place on a roller at 37°C for at least 24h. Initially a

considerable amount of the citrate dissolved in the acetic acid, however with time, a white material precipitates out of the solution. This same phenomenon was also observed at room temperature (RT), however a longer time period is required. After this time, the reaction tubes were centrifuged at 48000rpm for 15 minutes. The clear supernatant was decanted and 10ml of fresh acetic acid was added to the white precipitate. The tube content was mixed very vigorously and the mixture was incubated at 37°C for at least 2h. The reaction tube was centrifuged as before and a further 10ml of acetic acid was added to the white precipitate and mixed vigorously. The pellet (pre-treated Na3Citrate) from this step is to be used in the synthesis of the compounds of the present invention. Just before use, the tubes were centrifuged and the clear supernatant was removed.

The proposed structure of Nephronin is a platform for synthesising an array of esters of citrate, where different fatty acid molecules are esterified to the hydroxyl group on the citrate molecule. In this instance, essentially five such acids are used in the synthesis procedure to demonstrate the validity of the synthesis and the bioactivity of the products.

Phosphorus oxychloride ($POCl_3$) was chosen to convert the fatty acids to acid chloride (R-C=O-Cl). The acid chloride when added to the citrate should then form an ester with the –OH group on carbon 3 of the citrate molecule.

Phosphorus oxychloride reacts violently with water to form hydrochloric acid (HCl). To prevent this reaction occurring, the entire procedure was performed in the absence of water, in hexane.

The following reaction tubes were set up for the formation of the acid chloride.

1. 8ml of Acetic Acid + 10ml of hexane + 1 ml of $POCL_3$
2. 33ml of Propanoic Acid + 10ml of hexane + 1 ml of $POCL_3$
2. 9ml of Butyric Acid + 10ml of hexane + 1 ml of $POCL_3$
3. 94ml of Hexanoic Acid + 10ml of hexane + 1 ml of $POCL_3$
4. 74ml of Octanoic Acid + 10ml of hexane + 1 ml of $POCL_3$

The tubes were quickly capped under nitrogen and incubated at RT on a roller for 30minutes. The content of each tube was added to appropriately labelled tubes containing the pre-treated Na_3Citrat. Once again each tube was quickly capped under nitrogen and the content of the tube was mixed vigorously until the entire Na_3Citrate pellet was fully mixed with the solvent

mixture containing the acid chloride of each fatty acid. The tubes were then incubated at RT on a roller over night.

For control reaction, 1.8ml of acetic acid was added to the pre-treated $Na_3Citrate$. To this mixture 10ml of hexane was added without the $POCl_3$. To compensate for the absence of $POCl_3$ and the fact that the major by product of $POCl_3$ would be phosphoric acid, 1ml of 80% ortho phosphoric acid was added to the mixture.

6. 1.8ml of Acetic Acid + 10ml Hexane + 1ml of H_3PO_4

The control tube was otherwise treated exactly the same as the reaction tubes.

After an over night incubation of the tubes at RT on a roller, the tubes were centrifuged at 48000rpm for 20min. In every case a solid pellet, a liquid phase and the upper solvent phase could be seen. Interestingly in the case of Acetic Acid reaction tube, the lower liquid phase accounted for approximately 5-7ml, whereas in the case of Octanoic Acid, the lower liquid phase only accounted for 1-2ml.

In each case the upper solvent phase was decanted. To each tube 10ml of fresh hexane was added and the content of each tubes was mixed by shaking very vigorously followed by centrifugation as before. The solvent layer was decanted and the washing procedure was repeated twice more. It is noteworthy that with each hexane washing step, the liquid lower layer was reduced in size and the size of the solid precipitate proportionally increased in size.

After the washing procedure, the pellets were dissolved in a 10% solution of ammonium hydroxide and the pH of the samples continuously monitored and the pH of the samples adjusted to pH 7.2-7.4. In each case the volume of the samples were adjusted to 20ml with milli-Q water.

To remove the phosphoric acid and the un-reacted citrate, an excess of $CaCl_2$ was added with the addition of 13ml of 2.5M solution of calcium chloride ($CaCl_2$) to each tube. The tubes were mixed very vigorously to allow the precipitation process to complete. The tubes were capped and placed on a roller over night. After this time the tubes were centrifuged to remove the precipitate. In each case the supernatant was decanted into appropriately labelled tubes. To the pellets, 5ml of water was added, the tubes were mixed thoroughly, the tubes were re-centrifuged and in each case the resulting supernatant was added to the tubes containing the supernatant from the earlier centrifugation. It is understood that a considerable amount of the compound might also be

precipitated by this step, however this was found to be necessary for the purification of the product free from Na_3Citrate and the structural conformation of the products.

To precipitate the excess $CaCl_2$ in the samples, excess saturated solution of freshly prepared ammonium bicarbonate was added to each tube. The resulting precipitates were removed by centrifugation at 48000rpm for 10min. The samples were dialysed using a dialysis membrane with a cut off of 100dalton. Large volume of water with regular changes was used for dialysis with stirring. After extensive dialysis (3 days), the samples were centrifuged and the clear supernatnats were decanted. The pH of the samples (supernatnats) were checked to ensure that the pH of the samples were within pH 7.0-7.4. The samples were freeze-dried and the weight of the lyophilised product was estimated in each case. The samples were then dissolved to 1mg/ml in milli-Q water and filter sterilised using 0.2μ sterile membranes prior to biological testing of the samples. In each instance the volume of the product was approx. 40ml. In the case of the control preparation very little lyophilisate was found. In this instance the volume of the control preparation was adjusted to 40ml similar to the volume of the samples.

These preparations are referred to hereafter as prearations 1 to 5.

It should be noted that these preparations also contain other material other than the compounds of the present invention. Consequently the effective amount of the compounds of the present invention used in the biological studies is considerably lower than indicated. The filter sterilised preparations were used as stock for testing samples for testing the biological activity.

Structural conformation of the synthesised material.

The five preparations were subjected to structural analysis. Each preparation derivatised by various esters contained two compounds. These were the 2-acetyl- and 2-alkanoyl-Citrate (where the "alkanoyl" was propanoic, butanoic, hexanoic or octanoic acid). This assignment was based on 2D NMR spectroscopy, particularly HMQC spectrum and the mass spectrometric analysis of each component. No free citrate was observed but there was some free acetate in each sample.

Urinary Factor

SRNS also known as Minimal Change Nephrotic Syndrome (MCNS) is the most frequent form of nephrotic syndrome in children, with the peak incidence at 3-5 years of age. The aetiology

and pathogenesis of SRNS are unknown. Patients with SRNS characteristically have normal glomerular filtration with little or no change in the kidney biopsies under light microscopy (53; 54). However, the fusion of the foot processes is only observed with the use of electron microscopy (55). Focal Segmental Glomerular Sclerosis (FSGS) shares many clinical and pathological features with SRNS (56; 57), and its peak incidence occurs in older children and young adults. FSGS is thought to be the end stage renal disease form of SRNS, and patients with FSGS do not often respond to steroid treatment (58).

Given the fact that the kidney has been shown to function both as a size and a charge selective barrier (59; 60), and that the glomerular basement membrane (GBM) and the foot processes of the epithelial cells are covered by negatively charged heparan sulphate glycoproteins (61) (62), the reduction in the anionic sites is thought to greatly contribute to loss of albumin in the urine (63).

On the basis of clinical observations; 1) Association of the remission of lipoid nephrosis with natural measles, 2) Induction of remission by steroids that is prolonged by treatment with cyclophosphamide, 3) Occurrence of similar glomerular disorders in Hodgkin and non-Hodgkin's lymphoma diseases, circulating factors produced by systemic abnormality in T-cells were thought to play an important role in the pathogenesis of SRNS (64). This was supported by experiments where infusion of plasma from SRNS patients into rabbits, caused a reduction in the number of anionic sites and the concomitant increase in proteinuria (65). There are at least two documented proteinaceous factors associated with SRNS, vascular permeability factor (66-68), and soluble immune response suppressor (69-72). Further, abnormalities in the cellular immune system, followed by evidence illustrating impaired T-lymphocyte colony formation (73), depressed lymphocyte blast formation with some T-cell mitogens (74-76), supports an immune mediated disorder.

The present inventor's initial work on SRNS concentrated on the purification of a postulated circulatory highly cationic protein (77) that was said to neutralise negatively charged heparan sulphate leading to proteinuria. The fact that heparan sulphate is the major glycosaminoglycan coating the glomerular basement membrane, producing an electrostatic charge barrier, made the concept of a circulatory highly cationic protein an attractive one.

The established methodology for the detection of glycosaminoglycans (GAGs) was based on the precipitation of highly anionic GAGs with cationic dyes and detergents. The methodology developed by Whiteman (78; 79) with minor modifications (77) using the cationic dye Alcian Blue for the precipitation and quantification of GAGs was used for the detection of this hypothesised cationic protein. A successful purification procedure from the urine of children with SRNS was established. SDS-PAGE analysis of this protein showed two bands, a 50kDa and a 25kDa molecular weight band, under reducing conditions. The amino acid sequencing data from these bands suggested that the purified protein was of immunoglobulin origin (Data not shown).

Modified flat-bed cellulose acetate electrophoresis was used for the detection of material in chromatographic fractions that might bind to heparin and inhibit its migration (47). Using this methodology, it was first noticed that fractions with little or no protein could potently inhibit migration of heparin. Initial analysis showed the presence of non-esterified fatty acids, although this activity was not related to the lipid portion. To demonstrate the specificity of this compound for heparin, its effect on the migrations of a number of GAGs were tested. The specific binding of this factor to Heparin and heparan sulphate but not chondroitin and dermatan sulphate was observed. The inhibition in migration of heparin and heparan sulphate is ascribed to specific interaction of the factor with these molecules, rather than on the basis of charge, since all these molecules are highly negatively charged. Histochemical staining of human umbilical vein endothelial cells (HUVEC) (42; 43) clearly demonstrate the binding of the factor with heparan sulphate covering HUVEC (49) showing considerable reduction in the amount of staining for heparan sulphate, compared to untreated cells. In order to test the effect of this factor on cell surface heparan sulphate of viable endothelial cells, the factor was added to the culture medium. Initial experiments showed that exposure of the viable cells to the factor caused the dissociation of the cells from the substratum. To minimise the dissociation of the cells from the substratum, the incubation period was reduced from 1 hour to 10min. The cells were then stained for surface anionic sites as above.

Dissociation of the cells from the substratum caused by the addition of the factor was not due to cell death since trypan blue staining of the cells after treatment with this material, showed over 95% cell viability. Addition of the cells to fresh culture plates after removal of the factor by a washing process with PBS, resulted in re-adherence of the cells to the plate. This suggests that the

binding of the factor to heparan sulphate on the cell surface and possibly the extracellular matrix is responsible for separation of the cells from the substratum.

The fact that this compound tightly bound to anion exchange columns during the purification procedure indicated that, overall Nephronin exhibits a negative charge. Consequently, its binding to negatively charged molecules such as heparin and heparan sulphate, but not other GAGs, is site directed rather than charge. Given the apparent negative charge displayed by this factor and its affinity for lipids, suggested that it might behave as a cationic detergent with specificity for certain sugar moieties. It was therefore hypothesised that like polymyxin B this compound might have an LPS neutralising ability. Utilising the enzymatic assay, Limulus Amoebocyte Lysate (LAL) assay system (80) for the detection of LPS, I was able to demonstrate that this compound was a potent inhibitor of LPS. Table 1 shows a typical endotoxin neutralising experiment utilising the LAL assay.

This factor was named "Nephronin" to reflect the source and the origin of the material. The inhibition of LPS by Nephronin in the LAL assay is thought to be due to chemical modification of the lipid A moiety by Nephronin. Further studies showed that Nephronin could agglutinate Gram-negative but not Gram-positive bacteria (Table 2). This suggests that Nephronin binds directly to the LPS molecule on the bacterial cell surface, however the fact that Nephronin with molecular weight less than 1kD can agglutinate Gram-negative bacteria, is of particular interest.

The interaction of Nephronin with LPS might prevent the binding of LPS to LBP, thereby inhibiting cytokine production by MØ and other LPS responsive cells. Preliminary studies showed that Nephronin inhibited LPS-induced IL-4 and IL-6 by fibroblasts.

Having established Nephronin's ability to bind to heparin and heparan sulphate, it was important to establish whether this material had any effect on blood coagulation. A commercially available chromogenic kit for the measurement of heparin like molecules, based on the acceleration of antithrombin III activity for the inhibition of thrombin formation was used (50; 51). Nephronin seemed to behave in much the same way as heparin and in synergy with heparin. These findings further support the notion that Nephronin may self-polymerise, since activation of antithrombin III requires at least a pentasaccharide sequence of heparin (52). Both extrinsic, UPTT and intrinsic, KPTT were performed as directed by the suppliers (Diagnostic Reagents Limited, Thames, Oxon. UK). Nephronin was found to prolong coagulation time in much the same way as heparin. Contrary to expectations, the anti-coagulation effects of Nephronin were found to be in synergy with heparin.

Identification of cells of origin

Although the pathogenesis of SRNS is still unknown, it is well known that patients with SRNS go into relapse with upper respiratory and minor infections. As mentioned earlier, the loss in immune regulation is thought to play an important part in the pathogenesis of the disease. To test whether the immune cells from SRNS patients during relapse produce (secrete) Nephronin without mitogen stimulation, peripheral blood mononuclear cells (PBMC) from SRNS patients were isolated. To maintain the cells, 50units/ml of IL-2 were added to the culture media. The cell culture supernatant was removed at time intervals and processed analytically for isolation of Nephronin. To examine the presence of circulatory compounds that might stimulate production of Nephronin, PBMC from normal subjects were cultured in media containing 10% SRNS patient's plasma, and the culture supernatant processed as above and the presence of Nephronin determined semi-quantitatively. The data presented in Table 3 clearly demonstrates that, lymphocytes isolated from SRNS patients during relapse are capable of producing Nephronin. Further, addition of patients' plasma, but not plasma from normal subjects, to cultures of lymphocytes from normal subjects also induced these cells to produce Nephronin. The fact that Nephronin alone has an autocrine ability together with its anti-LPS ability suggests that Nephronin is an immune related compound and that the pathogenesis of SRNS is solely due to lack of immune regulation. To test this hypothesis, urine from three children with meningitis and two normal children were processed for analytical isolation of Nephronin. Nephronin was isolated from the urine of two of the meningitis patients who had survived the disease. The urine of the patient that did not contain Nephronin did not survive the disease.

Nephronin was not isolated from the urine of normal children.

The synthesised Nephronin

As mentioned above, studies had indicated that the urinary factor Nephronin is an ester of citrate formed from a heterogeneous mixture of fatty acids. On this basis a number of Nephronin-like compounds were synthesised using the same methodology. Fig 1 shows the inhibition of LPS-induced TNF-α and IL-1α by five different compounds of the present invention. Human PBMC were first mixed with 1ng/ml of LPS, prior to being added to 24 well tissue culture plates, containing different amounts of the compounds of the present invention. The cells were harvested at 24, 48 and

72h post-stimulation and the concentration of the cytokines determined by sandwich ELISA in the culture supernatants. The dose dependent inhibition profile for these preparations of the compounds of the present invention clearly demonstrates the potency of these compounds in neutralising the ability of LPS to activate macrophages, to secrete TNF-α and IL-1α.

The ability of five compounds of the present invention and a control preparation to bind to heparin and inhibit its migration on cellulose acetate electrophoresis was tested. As expected the control preparation does not show any effect on the migration of heparin. However, preparations 1 to 5 showed an increasing ability in their inhibition of migration of heparin. This closely correlates to the increasing carbon chain length of the compounds of the present invention, where preparation 1 has the shortest chain length and preparation 5 the longest.

Compounds of the present invention were tested for their ability to inhibit the activity of human platelet heparanase (81; 82). Fig 2 shows the dose dependent inhibition of heparanase activity. Given the fact that heparanase activity is shown to be of particular importance in tumour development and metastasis, the ability of the compounds of the present invention to inhibit heparanase activity might suggest that the compounds of the present invention could play an important role in the inhibition of metastasis and inhibition of tumourogenic cells to solid tumours.

Normal and tumourogenic cell lines.

The importance of heparan sulphate in cell-cell interaction is becoming clear (83; 84). Furthermore, recent evidence suggests that tumourogenic cell surfaces might be covered by heparan sulphate that is different from that found on the surface of normal cells (85). To test the hypothesis that Nephronin might distinguish between the heparan sulphate covering the surface of normal and cancer cells, a normal fibroblast cell line (BFT-3B) and a cancer cell line (HT1080) were used. The cells were grown to confluence (single layer cells) in 24 well culture plates. Prior to the addition of the compounds of the present invention, the appearance of the cells was studied microscopically. Five different preparations of the compounds of the present invention with increasing carbon chain length were used in this study. Increasing volumes of different preparations were added to the cells and the cells were observed immediately after the addition of samples.

In the case of carcinogenic (HT1080) cell line, the cells contracted immediately after the addition of Nephronin, although they seemed to regain their original shape after a few hours.

However, within 24 hours incubation in media containing the compounds of the present invention, development of large vacuoles within the cells could be seen. After this stage, over 90% of the cells were found to be dead. In the case of normal (BFT-3B) cell line, addition of the compounds of the present invention did not seem to have any effect on the cells. This clearly demonstrates that the compounds of the present invention are highly toxic to cancer but not normal cells.

To test the effect of synthesised compounds of the present invention on other cell types, normal mouse muscle cell line (C2C12) and mouse neuron tumourogenic cell (B35) lines were used. Prior to the addition of the compounds of the present invention, the appearance of the cells was observed microscopically. In the case of C2C12 cell line, the cells had a characteristic dendritic appearance, with long protruding tentacles. The B35 cells of the neuron linage showed a characteristic appearance of nerve cells with long tentacles associated with such cells.

Increasing amounts of the 5 compounds of the present invention prepared were added to the cells. The cells were monitored on a regular basis for any changes to their appearance and whether they separated from the substratum. Shortly after the addition of the compounds of the present invention, the cells changed shape very quickly giving a round spherical appearance, irrespective of the amount of the compound added to the cells, although they remained bound to the culture plates. After 2h incubation with the compounds of the present invention, the cells separated from the substratum forming large clumps. The cells were incubated in media containing the compounds of the present invention for five days. Incubation of B35 cells with these preparations of the compounds of the present invention, after 24h resulted in the appearance of small vesicles, which was ascribed to cell lysis. This effect was much more pronounced with larger volumes of the compounds of the present invention. However, after 48h incubation of the cells, this dose dependent lysis could not be distinguished. Almost total cell death was observed after 96h incubation of B35 cells with any one of the five preparations. It is noteworthy that preparations of the compounds of the present invention with longer chain length seem to be slightly less toxic to the cells. After the five-day incubation period, the cells were washed free of the compounds and placed in fresh culture plates. In the case of C2C12, the cells remained viable and re-adhered to the plate, without regaining their original shape.

Given the fact that preparations of the compounds of the present invention were toxic to B35 and HT1080 but not C2C12 and BFT-3B suggests specific interaction of the compounds of the present invention with cell surface molecules on the cancer cell lines.

Adhesion of pathogenic *E.coli* to a gut cell line.

An experimental model to investigate the invasion of pathogenic *E.coli* was utilised to study the effect of the compounds of the present invention. Enteropathogenic *E.coli* (EPEC) carry virulence factors in the form of adhesins including plasmid-encoded fimbriae and a chromosomally born adhesin. These adherent *E.coli* alter epithelial cell morphology and the cytoskeleton of host cells (in this model a T84 epithelial cell line) as a mechanism to allow effective attachment and thus colonisation. Infection of confluent T84 cells with EPEC usually results in bacterial adherence, measured by a bacterial binding assay.

The cells were then inoculated with 10^7 colony forming units (CFU) of EPEC. Preliminary experiments had shown that addition of the compounds of the present invention to the inoculated cells, reduced the extent to which the media had become acidic, compared to inoculated cells that were not treated with the compounds, indicated by the change in colour of the media. However after approximately 3h incubation, the colour of the media had begun to change to yellow indicating that the media was becoming acidic. Consequently, after a three hour incubation of the treated epithelial cells with the inoculum, a second dose of the compounds of the present invention was added to the cells and incubated for a further three hours. At the end of the 6 hours infection period, cells were washed to remove any non-adherent bacteria. The cells were then lysed and bacterial CFU were counted at the dilutions that yielded between 25 – 250 CFU.

Addition of the compounds of the present invention to the epithelial cells inhibited bacterial adhesion and invasion of the cells by over 60%. Interestingly, addition of the compounds to bacteria alone did not inhibit bacterial growth, suggesting that the compounds do not have a bactericidal activity. Further, addition of the compounds to epithelial cells alone did not cause separation of cells from the substratum.

Herpes Simplex Virus (HSV)

Treatment of keratinocytes with the compounds of the present invention for 24h. followed by addition of virus inoculum to cell in fresh media inhibited HSV infection by 25-30%, determined by plaque reduction assay. However, when the compounds were added simultaneously or at 24, 48 and 72 hours after the addition of the virus inoculum, it inhibited virus infectivity by 100% (Fig 3). This suggests that pre-incubation of cells with the compounds results in the binding of the compounds to the cell surface heparan sulphate. The compounds are thought then to be taken up by the cells and degraded and the cell surface heparan sulphate regenerated. However, the fact that the compounds totally blocks infection of keratinocytes by HSV, suggests that the compounds preferentially bind to the site for binding to heparan sulphate on HSV. This binding clearly inhibits the ability of HSV to infect the keratinocytes.

Herpes simplex viruses establish lifelong latent infections in the sensory neuron of the host dorsal root ganglia (DRG) where they undergo periodic reactivations. An *in vitro* model consisting of human DRG neurones and autologous epidermal cells in two separate chambers to study anterograde axonal transport of HSV1 was established. HSV1 infection of the human DRG neurone results in separate axonal transport of glycoproteins and nucleocapsids, which are likely to assemble into mature virions before crossing the intercellular gap between axonal termini and epidermal cells. In this system, glycoprotein and nucleocapside antigens are detected in the epidermal cells by immunohistochemistry and confocal microscopy at 20 hours post-infection of DRG (86). Subsequent development of HSV1 cytopathic plaques can be observed over the next 48 hours. This system was utilised to study the ability of two preparations of the compounds of the present invention to inhibit the transmission of HSV from human axon to epidermis in comparison to direct infection of epidermal infection. Fig 4 clearly shows that addition of the compounds to epidermal cells side of chambers simultaneously or 12 hours after infection of DRG with HSV1 significantly inhibits infections of epidermal cells. Interestingly preparation 5 seems to be more potent at inhibiting HSV infection of the epidermal cells. The only difference between the two preparations of the compounds used in this study is the number of carbon chain length used in the synthesis of the compounds. Similarly preparation 5 also show a significant (25%) inhibition HSV infection of epidermal cells at 18 hours post infection. These findings suggest a preventative role for the compounds of the present invention in controlling the spread of herpes simplex virus. Further studies are being carried out to

test whether the compounds of the present invention are able to inhibit infection of neuronal cells by HSV. Given the fact that the compounds of the present invention are a relatively small molecules, it is possible for the compounds of the present invention to cross the cell membrane barrier and bind directly to the internalised virus.

5

HIV virus

HIV virus binds to the cell surface of cells by a virus glycoprotein GP120. The binding of GP120 to the cell surface receptors, CCR5 and to a lesser extent CCR3 and CCR2, is the first step in HIV infection of blood or tissue derived macrophages (87). However, there is evidence that heparan sulphate as well as other cell surface sugar moieties indirectly play a role in HIV infectivity.

On the basis that the compounds of the present invention bind to heparin and heparan sulphate as well other sugar moieties, suggested that the compounds of the present invention might be able to inhibit HIV virus infection of macrophages. Alternatively, since the compounds bind to a host of sugar moieties, it might also be able to bind directly to glycoproteins, such as GP120 and inhibit infectivity of cells by the HIV virus.

Adherent macrophages were prepared from human blood. The cells were either pre-treated with preparations of the compounds of the present invention 24h prior to addition of HIV inoculum, or the compounds were added simultaneously with the HIV inoculum. The cells were then incubated with the HIV inoculum for 4h, prior to being washed and fresh media added. The concentration of extracellular p12 antigen and levels of intracellular HIV DNA measured by quantitative PCR determined the susceptibility of macrophages to HIV infection.

Pre-incubation of macrophages with micro molar concentrations of the compounds resulted in the separation of the cells from the substratum. However, the cells remained viable as demonstrated by Trypan blue staining. Addition of HIV inoculum to macrophages pre-treated with the preparations of the compounds of the present invention did not inhibit HIV infection of cells. However, where it was added simultaneously with the HIV inoculum, up to 30% inhibition of HIV infection was observed. It is noteworthy that even at micro molar concentrations of Nephronin added simultaneously with HIV inoculum, the macrophages did not separate from the substratum.

Animal Studies.

To date only a limited amount of animal studies have been performed, mainly to demonstrate that the compounds of the present invention are not toxic to animals. Up to 200µl of each of the preparations were injected either subcutaneously or intrapartonially to mice ranging in weight from 20 to 25grams. When the purified analogues were injected to the animals they did not display any weight loss and behaved similarly to control animals treated with saline.

The data presented here describes a low molecular weight, non-proteinaceous compound that is produced by human lymphocytes when appropriately stimulated. The presence of Nephronin in the urine of children with nephrotic syndrome might suggest that therapeutic use of this compound might lead to induction of Nephrosis. However, given the fact that Nephronin was also isolated from the urine of children with bacterial meningitis suggests that Nephronin can be produced by the immune cells in response to infection. However its presence in the urine of children with SRNS is thought to be due to lack of immune down regulation which results in the observed Nephrosis. The discovery and synthesis of the compounds of the present invention presents a set of new therapeutic agents for the treatment of a number of infections diseases such as sepsis and herpes virus and other viruses that use similar mechanism in their pathogenesis. Further, since Nephronin potently inhibits production of cytokines such as TNF-α, IL-1α, it can potentially be used as a therapeutic agent in diseases that involve these cytokines, such as rheumatoid arthritis and similar immune related diseases. The ability of Nephronin to interact specifically with heparin and heparan sulphate indicates a potential use for analogues of Nephronin for the inhibition of tumour growht and metastasis.

The data shown for HSV clearly show that Nephronin inhibits infection of cells by herpes virus and as such an immediate therapeutic use for Nephronin is a vaginal cream as a preventative measure for the spread of HSV.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. All references referred to herin are incorporated by cross-reference.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing

from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Table 1. Endotoxin Neutralising Activity of the Nephronin utilising the LAL assay.

	Additions		OD @ 405nm
Positive Control	Proenzyme	Endotoxin	1.2
Negative Control	Proenzyme	Pyrogen Free water	0.3
Sample	Proenzyme	Endotoxin + Nephronin	0.02

The values shown in the table are a set of representative data obtained using the LAL assay. Where pyrogen free water was used for negative control studies, there is persistent low level activation of the pro-enzyme as shown by the relatively high background values. This is thought to be due to minor contamination with endotoxin. The data clearly demonstrates that Nephronin effectively neutralises endotoxin.

Table 2. Binding of Nephronin to Gram-negative endotoxin and its agglutinating effect.
5µl of the organisms were mixed with either 5µl of Nephronin or 5µl of PBS on a glass slide. After mixing the cells, the time taken for the agglutination of the cells were recorded. Where the factor was added, agglutination occurred within 2 minutes.
For control studies, Gram-positive bacteria; *S. aureus* and S. *epidermidis* were used. These studies were carried out using either heat killed and/or penicillin killed or viable organisms.

	Viable Organisms		Heat and/or penicillin killed	
Organisms	Organism + PBS	Organism + Nephronin	Organism + PBS	Organism + Nephronin
E. coli	-	+	-	+
Pseudomonas aeruginosa	-	+	-	+
N. meningitidis				
A	-	+	-	+
B	-	+	-	+
W135	-	+	-	+
S. aureus	-	-	-	-
S. epidermidis	-	-	-	-

-. No agglutination was observed

+. Agglutination observed

It is important to point out that agglutination of Gram-negative bacteria with Nephronin was not always clearly visible. However, effective agglutination could be achieved by using higher concentrations of Nephronin.

Table 3. Identification of cells of origin for the production of Nephronin.

Peripheral Blood Mononucleocytes (PBMC) were isolated from SRNS patients and Normal Subjects.

The cells were cultured in medium containing IL2 to maintain the cells.

	Source	Additions	Assay for Nephronin
PBMC	SRNS	Nil	Positive
PBMC	Normal	Nil	Negative
PBMC	Normal	10% SRNS Plasma	Positive
PBMC	Normal	10% Normal Plasma	Negative
PBMC	Normal	Nephronin	Positive

The data presented here demonstrates that PBMC from SRNS patients during relapse are capable of producing Nephronin. Further, sera from these patients stimulated PBMC from healthy subjects to produce Nephronin. Given the fact that Nephronin alone can stimulate PBMC from normal subjects, suggests that Nephronin can stimulate the immune cells in producing Nephronin in an autocrine fashion.

These PBMC were cultured for periods of up to 72 hours and at 24 hour intervals the culture media were removed and tested for the production of the Nephronin.

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